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Assessment of Retrovirus-Expressed Nucleoprotein as a Vaccine against Lethal Influenza Virus Infections of Chickens

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SUMMARY. Hemagglutinin-based influenza vaccines stimulate protection in chickens that is limited to the serotype of the expressed hemagglutinin. To evaluate whether a more highly conserved influenza virus protein might stimulate a broader protective response, the influenza virus nucleoprotein (NP) was introduced into a retroviral vector (mRCAS/NP). NP is an internal influenza virus protein that has been shown to stimulate cytotoxic T-cell responses in influenza-virus-infected mice. Cells infected with mRCAS/NP expressed approximately 10% of the level of NP observed in influenza-virus-infected chicken embryo fibroblasts. Immunocompetent chicks were vaccinated intramuscularly with ~1 × 10^5 NP-expressing units of mRCAS/NP. Four weeks later, chicks were bled and challenged with a highly pathogenic avian influenza virus (A/Chicken/Victoria/1/85). The NP-expressing vector stimulated an influenza-virus-specific response, as indicated by the presence of antibody to NP, but failed to protect against the lethal challenge.

RESUMEN. Evaluación de la nucleoproteína expresada por retrovirus como una vacuna contra las infecciones con el virus de la influenza en pollos.

Las vacunas de influenza basadas en la hemoaglutinina estimulan una protección en pollos que se limita al serotipo de la hemoaglutinina expresada. Para evaluar si una proteína altamente conservada del virus de la influenza podía estimular una respuesta protectora más amplia, la nucleoproteína del virus de la influenza se introdujo en un vector retroviral (mRCAS/NP). La nucleoproteína es una proteína interna del virus de influenza que estimula la respuesta citotóxica de las células T en ratones infectados con el virus de influenza. Las células infectadas con el vector expresaron aproximadamente el 10% del nivel de nucleoproteína observada en fibroblastos de embrión de pollo infectados con el virus de influenza. Se vacunaron intramuscularmente aves inmunocompetentes con ~1 × 10^5 unidades del vector. Cuatro semanas más tarde los pollos se sangraron y se desafiaron con un virus de influenza aviar altamente patógeno (A/Pollo/Victoria/1/85). El vector que llevaba la nucleoproteína estimuló una respuesta específica para el virus de influenza como lo indicó la presencia de anticuerpos contra la nucleoproteína, pero no protegió contra el desafío letal.

Attempts to develop subunit vaccines to avian influenza virus have concentrated on the viral envelope glycoproteins hemagglutinin (HA) and neuraminidase (NA). Viral vectors expressing these glycoproteins have successfully stimulated protection against lethal influenza virus challenges (5,9,21,22). Unfortunately, the high variability associated with the HA and NA proteins have limited this protection to influenza viruses with the same HA or NA serotype used for immunization.

In the present study, in order to evaluate whether more-conserved influenza virus proteins might be able to elicit immune responses that could protect against infection with viruses of different HA and NA serotypes, we have expressed the influenza virus nucleoprotein (NP) in an avian leukosis virus vector. NP is a conserved internal virus protein that has been shown

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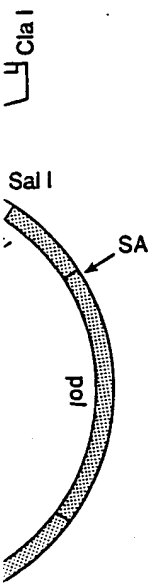

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Enzyme-linked immunosorbent assay (ELISA) for NP. To evaluate the level of expression of the inserted NP gene, chicken embryo fibroblasts (CEFs) were infected with undiluted virus stock harvested from transfected turkey embryo fibroblasts. One week later, after the non-cytopathic vector spread throughout the culture, an ELISA comparing the amount of NP present in mRCAS/NP-infected CEFs relative to the level of NP expressed in influenza-infected CEFs was performed on lysates from the infected cells. Wells were coated with an NP-specific monoclonal antibody (MAb) (anti-WSN 7/3) and blocked with bovine serum albumin (BSA). Cell lysates (prepared in 1% Triton X-100, 0.33% deoxycholate, 2 mM PMSF) were diluted to tissue protein concentrations of 50 μg/ml in phosphate-buffered saline (PBS) containing 1% BSA and 0.1% Tween 20 (Sigma Chemical Co., St. Louis, Mo.). A second NP-specific MAb, anti-WSN 5/1, was biotinylated by mixing 1 mg of MAb (in 1 ml 0.1 M $NaHCO_3$) with 120 μg of *N*-hydroxysuccinimide biotin ester (Sigma) in dimethyl sulfoxide at 1 mg/ml and incubating for 4 hours at room temperature. Free biotin was removed by extensive dialysis against PBS. Bound NP was detected using the biotinylated MAb followed sequentially by a streptavidin-alkaline phosphatase conjugate (Gibco BRL, Grand Island, N.Y.) and *p*-nitrophenyl phosphate (Sigma). Serial dilutions of a lysate prepared from CEFs 9 hours after infection with influenza virus (A/Seal/Mass/80) served as a standard of reference.

ELISA for antibodies to NP. This ELISA was performed as described (11) on plates coated with A/Turkey/Ireland/1/83 (Ty/Ire/83) (H5N2), a virus with avian influenza A-specific NP antigen.

Assessment of vector titers and insert stability. Previous experience with HA expression in an ALV vector demonstrated that HA sequences were lost with serial passage of the vector stock (9). Titers of the vector and insert stability during serial passage of the NP-expressing vector (mRCAS/NP) were determined using an immunostaining procedure described by Stoker and Bissell (17). Virus stocks obtained from transfected turkey embryo fibroblasts or after a single passage of a virus stock on CEFs were evaluated for NP expression on the quail cell line QT6 (13). QT6 cells were infected with dilutions of test stocks. Three days later, foci of cells expressing the avian leukosis virus capsid protein p27 or influenza NP were detected using MAbs specific for either p27 (9Q, obtained from G. F. de Boer, Central Veterinary Institute, Lelystad, The Netherlands) or NP (anti-WSN 5/1).

Experimental design. Vaccination and challenge studies were performed in P3 facilities. The ability of avian-leukosis-virus-expressed NP to stimulate a protective immune response was evaluated by immunizing chicks with ~1 × 10^5 NP-expressing units of mRCAS/NP. Chicks immunized with ~1 × 10^5 H7-expressing units of RCAS/H7 served as a positive control, and chicks immunized with a similar number of infectious units of mRCAS served as a negative control. At 3½ weeks of age, chicks were bled and inoculated intramuscularly with 0.1 ml of undiluted vaccine (~10^5 NP- or HA-expressing units) or control (mRCAS) stocks. Four weeks later, serum was collected, and chickens were challenged via the nares with 0.1 ml of a 1:100 dilution of A/Chicken/Victoria/1/85 (H7N7) stock (approximately 10^6 mean egg infective dose). Tracheal and cloacal swabs were collected 3 days later, and samples were tested for the presence of replicating influenza virus by inoculation into 11-day-old embryonated eggs. Surviving birds were bled and killed 2 weeks after challenge.

This protocol was repeated in a second trial involving 11 mRCAS-inoculated chickens and 11 mRCAS/NP-inoculated chickens. Levels of influenza virus in lung homogenates of terminal chickens were determined by inoculating 11-day-old embryonated eggs with serially diluted samples.

RESULTS

Level of expression and stability of NP insert. An ALV vector expressing the NP gene from an influenza A virus (A/Chicken/Pennsylvania/1/83) was constructed as described above (Fig. 1). The level of NP observed was approximately 10% that obtained following infection of CEFs, with a high multiplicity of infection of the influenza virus A/Seal/Mass/80. The relative level of NP expression by mRCAS/NP- and A/Seal/Mass/80-infected chicken cells was similar to that previously reported for HA expression by RCAS/H7 and Seal/Mass/80 (9).

Stocks harvested from the transfected turkey cells contained 5 × 10^5 to 1 × 10^6 vector and NP-expressing foci per ml. An approximately 90% reduction in the number of NP-expressing foci with no concurrent reduction in vector-expressing foci was observed following a single passage of mRCAS/NP on chicken cells. These results indicate that the NP gene, like the HA gene, is rapidly lost from the avian leukosis virus vector.

Response to vaccination. Four weeks post-vaccination, mRCAS/NP-inoculated chickens had detectable levels of antibodies against influenza NP, as determined by a direct binding ELISA using an influenza virus of the H5 serotype as the source of influenza virus antigens (Table 1). The lower serum response of RCAS/H7-immunized chickens to the H5 influenza vi-

to be a frequent target for cytotoxic T-cell responses of mice. Humoral responses to this internal virus protein do not provide protective neutralizing or opsonizing activity. In the murine influenza virus model, adoptive transfer of T-cells with cytotoxic activity for NP-expressing cells can protect against lethal infection (2,18). Furthermore, mice orally immunized with attenuated *Salmonella typhimurium* engineered to express NP mount strong T-cell responses to NP-expressing cells. Such mice are protected against a lethal influenza virus challenge if they have been boosted intranasally with recombinant NP protein (19).

Little research has been done on the use of NP to stimulate protective immune responses in chickens. In one study, a fowlpox virus was used as a vector to express NP. This vector elicited antibodies to NP but failed to protect against a lethal challenge (22). In the present study, we used an avian leukosis virus (ALV) vector to express NP. ALV vectors differ from fowlpox vectors in that: A) they do not kill cells and thus allow stable, long-term presentation of expressed antigens to the host immune system, and B) they express many fewer proteins favoring host responses against the expressed insert as opposed to the competing vector proteins. The objective of the present study was to determine whether an NP-expressing ALV vector would be more effective than a fowlpox vector in stimulating a protective immune response.

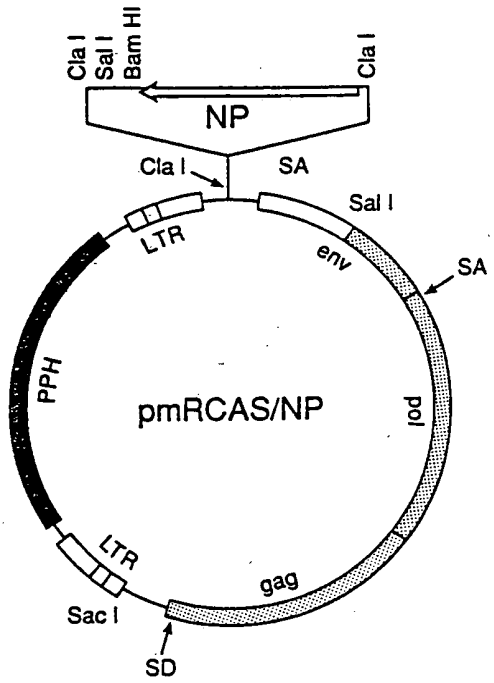


Fig. 1. Schematic of pmRCAS/NP. The open arrow at the top represents NP insert. The black portion of the circle represents plasmid PPH, and the shaded portion represents sequences from pRAV-1. LTR = proviral long terminal repeats; *gag, pol,* and *env* = retroviral structural genes; SD and SA = splice donor and splice acceptor sequences, respectively. For construction, see Materials and Methods.

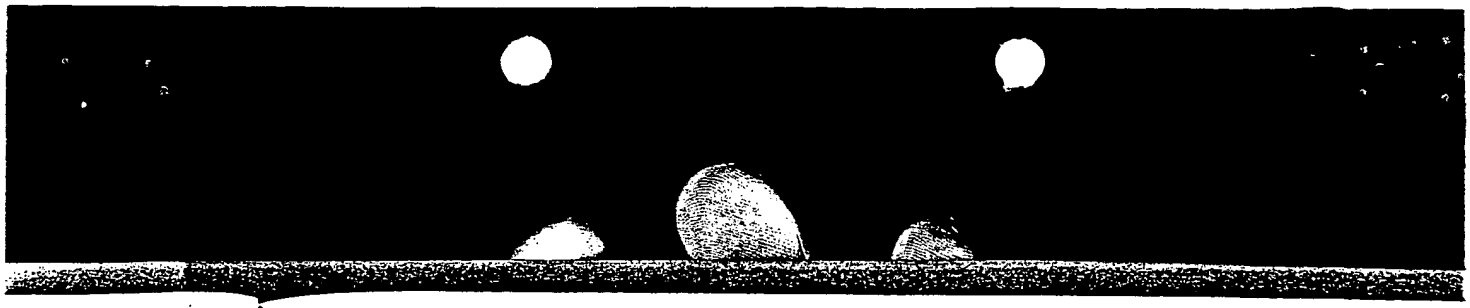
MATERIALS AND METHODS

Chickens. Specific-pathogen-free chickens were obtained from SPAFAS, Inc. (Norwich, Conn.). A total of forty-five 3½-week-old chickens were used in two vaccine experiments.

Plasmids. Plasmid DNAs used for vector construction included pNP33, a plasmid containing the NP gene of an influenza A virus (A/Chicken/Pennsylvania/1/83) (3); pRCAS, a plasmid containing a nonpermuted proviral form of a replication-competent, non-transforming derivative of the Schmidt-Ruppin-A strain of Rous sarcoma virus (7,8); pRAV-1, a plasmid derivative of pBR322 containing Rous-associated virus-1 DNA (15); and pCLA12N, a plasmid that facilitates introduction of fragments into the unique *ClaI* site of pRCAS (8). The pRCAS vector was modified by replacing the 5.8-kilobase *SacI* to *SalI* fragment encoding *gag, pol,* and *env* sequences with the homologous fragment from pRAV-1. Virus (mRCAS) rescued from this construct was more effective than RCAS in establishing persistent viremia in 1-day-old inoculated K28 chickens (unpublished observations). The construction of pRCAS/H7, an HA expressing vector, has been described (9).

Construction of NP-expressing vector. An approximately 1.6-kb *EcoRI-BamHI* fragment containing the entire protein-coding sequences of the NP gene from pNP33 was inserted into the multiple cloning site of pCLA12N. The NP gene, now bounded by a 3′ *ClaI* site in CLA12N and a 5′ *ClaI* site in noncoding sequences of the NP gene, was introduced into the *ClaI* site of pmRCAS generating pmRCAS/NP. The orientation of the insert was determined by digestion with *SalI* (Fig. 1).

Recovery of virus from plasmid DNA. Virus was recovered from pmRCAS/NP by transfection of early-passage turkey embryo fibroblasts using the DEAE-dextran procedure followed by a 20% dimethyl sulfoxide shock (10). Transfected cells were passed when they became confluent, and stocks (medium from in-



(chicken major histocompatibility complex) and thus representative of commercial lines. However, despite the fact that they were outbred, the tested chickens may not have had *B* haplotypes capable of presenting influenza NP sequences to T cells.

A third factor could reflect the fact that immunization with NP, an internal virus protein, does not produce humoral responses that can neutralize virus or clear virus by opsonization. For a rapidly spreading infection such as avian influenza, the presence of neutralizing or opsonizing antibodies may be essential to prevent an infection from overwhelming the host before cell-mediated killing of infected cells can provide protection.

NP has also been found to be much less effective than HA in vaccination trials with poxvirus-expressed immunogens. In the avian influenza model, vaccinia- and fowlpox-expressed HA can stimulate prolonged protection against influenza virus of the same HA serotype (5,22), whereas fowlpox-expressed NP has failed to protect (22). Similarly, in the mouse model system, vaccinia-expressed HA provides complete protection against challenge with the same serotype, whereas vaccinia-expressed NP does not protect against morbidity and has produced variable results in survivability (1,6,16).

In summary, retrovirus-expressed HA provides complete protection against influenza A virus of the immunizing serotype 9 months after a single immunization. Although retrovirus-expressed NP could potentially provide protection against challenge by many different HA or NA serotypes, the failure to elicit significant protection by a single immunization makes mRCAS/NP an impractical candidate for further development as an avian influenza virus vaccine.

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Table 1. Response of mRCAS/NP-vaccinated chickens to challenge with pathogenic influenza virus.

Treatment[A]	Protection[B] Sick/dead/total	Virus detection[C] Trachea	Virus detection[C] Cloaca	ELISA titer to Ty/Ire/83 (H5N2) Pre-challenge	ELISA titer to Ty/Ire/83 (H5N2) Post-challenge[D]
mRCAS/NP	5/5/6	5/5	4/5	1000	100,000
RCAS/H7	0/0/6	0/6	1/6	500	3000
mRCAS	6/6/6	5/5	5/5	<100	—
Control	5/5/5	4/5	5/5	<100	—

[A] A 0.1-ml intramuscular inoculation with ~1 × 10^5 infectious units at 3½ weeks of age.

[B] Two weeks after challenge with A/Chicken/Victoria/85 (H7N7) via the nares. No. sick chickens/no. dead/total per treatment group.

[C] Cloacal and tracheal swabs tested by inoculation into 11-day-old embryonated eggs. No. chickens testing positive/total tested.

[D] The high ELISA titer observed post-challenge for the mRCAS/NP-vaccinated group was obtained from a surviving bird.

rus probably represents some cross-reactivity between non-neutralizing epitopes of the HA proteins of the H5 and H7 serotypes. A titer of less than 100 observed with unvaccinated and RCAS-inoculated controls represents background binding.

Four weeks after immunization, each of the groups was challenged with the highly pathogenic avian influenza virus A/Chicken/Victoria/1/85. This virus has the H7 serotype. Only one out of six mRCAS/NP-inoculated chickens survived challenge (Table 1). In contrast, all six chickens inoculated with RCAS/H7 survived. The five control chickens inoculated with the vector without an influenza virus insert died. The one surviving bird from the mRCAS/NP-inoculated group had an extremely high post-challenge titer of anti-NP antibody (100,000, as opposed to 3000 for the RCAS/H7-protected group), indicating a secondary response to NP for the mRCAS/NP-immunized chicken. In the second trial, all mRCAS/NP-vaccinated chickens suffered morbidity, and similar numbers of mRCAS/NP- and mRCAS-inoculated chickens died within 10 days of challenge (data not shown). Furthermore, the levels of the challenge virus in lung homogenates were unaffected by immunization with mRCAS/NP.

DISCUSSION

The ideal vaccine for avian influenza should be effective without the need for boosting, should provide long-term protection, and should protect against a broad spectrum of influenza virus strains. Avian-leukosis-virus-expressed HA fulfills the first two requirements (9) but fails to protect against more than the immunizing serotype. A retroviral vector expressing NP was constructed in hopes of obtaining a vaccine that would provide protection against a variety of influenza virus serotypes. Unfortunately, the vector-expressed NP did not protect chickens against a lethal challenge, despite its ability to stimulate a serological response.

Several factors, either individually or in combination, could account for the fact that a single intramuscular injection with an NP-expressing retrovirus did not protect. First, the route of administration can affect the immune response to an antigen. Studies with influenza and rubella virus have demonstrated that the subcutaneous administration of virus leads to a significant stimulation of cell-mediated immunity in the systemic tissues and peripheral blood lymphocytes but little response in the respiratory tract. In contrast, intranasal or intrabronchial immunization induces a pronounced cell-mediated response in the respiratory tract with minimal responses in the peripheral blood (12,20). Therefore, the intramuscular route of inoculation in the present study may have failed to stimulate a rigorous cell-mediated response in the respiratory tract, where the response would have been most effective.

Second, the number of T-cell-stimulating epitopes associated with a protein are limited compared with epitopes capable of eliciting B-cell responses. Thus, in mice, T-cell responses to NP and the influenza polymerases, PB1 and PB2, occur only in association with specific major histocompatibility haplotypes (4,14). The SPAFAS chickens used in the present study are polymorphic at the *B* complex

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Differentiation of Pathogenic and Non-Pathogenic Serotype 1 Marek's Disease Viruses (MDVs) by the Polymerase Chain Reaction Amplification of the Tandem Direct Repeats within the MDV Genome

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SUMMARY. There are no simple, direct methods to reliably distinguish oncogenic serotype 1 Marek's disease viruses (MDVs) from their attenuated variants. The present study was an attempt to apply polymerase chain reaction (PCR) to develop a rapid and sensitive assay for the presence of the MDV genome. PCR oligos were chosen to flank the 132-base-pair tandem direct repeats in the serotype 1 MDV genome. The PCR reaction was specific for serotype 1 MDVs, amplifying fragments corresponding to one to three copies of the tandem repeats present in Md11/8, JM/102W, and GA viruses. A high-molecular-weight DNA smear was observed when the DNA from an attenuated Md11/100 was PCR-amplified. Use of the PCR technique allowed the detection of two copies of the 132-base-pair repeat in the DNA extracted from MDV-induced lymphomas removed from two chickens. No DNA was amplified from the DNA extracted from lymphomas induced by either an avian leukosis virus (RAV-1) or reticuloendotheliosis virus (chick syncytial virus).

RESUMEN. Diferenciación entre cepas patógenas y no patógenas del serotipo 1 del virus de la enfermedad de Marek, por medio de la amplificación por la reacción en cadena de la polimerasa de los segmentos duplicados (en tándem) del genoma el virus de la enfermedad de Marek.

No se han establecido métodos simples, directos y confiables para diferenciar las cepas oncogénicas del virus de la enfermedad de Marek del serotipo 1, de las variantes atenuadas. En el presente estudio se intentó aplicar la reacción en cadena de la polimerasa para desarrollar una prueba rápida y sensible para detectar la presencia del genoma del virus de la enfermedad de Marek. Se seleccionaron oligonucleótidos amplificados por la reacción en cadena de la polimerasa para flanquear los segmentos de 132 pares de bases del genoma del serotipo 1 del virus de la enfermedad de Marek. La reacción en cadena de la polimerasa fue especifica para el serotipo 1 del virus de la enfermedad de Marek, amplificando los fragmentos correspondientes de una a tres copias de los segmentos repetidos (tándem) presentes en las cepas Md11/8, JM/102W y GA. Se observó una fracción de alto peso molecular cuando se amplificó el ADN por medio de la reacción en cadena de la polimerasa de la cepa atenuada Md11/100. El uso de la técnica de la reacción en cadena de la polimerasa permitió la detección de dos copias del segmento repetido de 132 pares de bases en el ADN extraído de linfomas inducidos por el virus de la enfermedad de Marek de dos pollos. No se amplificó el ADN extraído de linfomas inducidos por el virus de la leucosis aviar (RAV-1) o por el virus de la reticuloendoteliosis (virus productor de sincitia en pollo).

Marek's disease (MD) is a lymphoproliferative disease of chickens characterized by lymphocytic infiltration of various organs. The causative agent is Marek's disease virus (MDV), a group of strongly cell-associated avian herpesviruses that have been subdivided into three serotypes. Serotype 1 viruses are the pathogenic viruses and their cell-culture-attenuated variants. Serotype 2 viruses are the naturally occurring non-pathogenic chicken viruses, and the non-pathogenic turkey herpesviruses, or HVTs, are designated as serotype 3 viruses (2,3).

The direct detection of MDV in the peripheral blood or tissue samples is not simple. Normally,

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